Laboratory studies on cancer chemoprevention by tea pigments.

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Summary
Tea pigments, the oxidative products of tea polyphenols, mainly contain theaflavins, thearubigins and other components. In short-term screening studies, tea pigments were found to have significant inhibitory effects in gene mutation test, micronuclei formation test, metabolic cooperation test, mouse ear edema test, viability and growth ability of Hela cells in soft agar and S-180 solid tumor test. In addition, in vitro studies showed that tea pigments induced quinone reductase (QR) activity and glutathione S-transferase (GST) activity in Hep G2 cells. The pigments were found to be effective in preventing formation of liver, colorectal and oral cancers in animal models. It was also found that oral administration of 0.1~0.5% tea pigments increased GST activity in rat liver by 18%, which was accompanied by significant increase of GST 1-1, 1-2, and 3-3 protein expression. Tea pigments inhibited the proliferating cell nuclear antigen labeling index (PCNA-LI), the expression of Bcl-2 protein and ras-p21 protein, and induced the expression of Bax protein in rat colorectal cancer model induced by dimethylhydrazine (DMH). PCNA-LI, silver stained nucleolar organizer regions (AgNOR) and epidermal growth factor receptor (EGFR) expression were also inhibited by tea pigments in the hamster oral cancer induced by dimethylbenzanthracene (DMBA). Our results suggest that tea pigments have significant chemopreventive effects on various animal cancer models, and the anti-cancer properties may be due to the activation of detoxifying enzymes such as QR and GST, the inhibition of cell proliferation and the induction of apoptosis.

Key words: tea pigments antioxidation cell proliferation apoptosis

Introduction
Many animal studies have shown that tea has strong protective effects on tumor formation in many organs, including colon, esophagus, liver, lung, mammary gland skin and oral cavity (1,2). Tea polyphenols are the major ingredients of green tea. It has been suggested that the most significant properties of tea polyphenols that might affect carcinogenesis are their antioxidant activities, modulation of immunity and activation of detoxification enzymes. Tea pigments are the major constituents of black tea. Recent studies demonstrated that theaflavins and thearubigins, the major constituents of tea pigments, are also strong antioxidants. Based on the multistage-theory of carcinogenesis (3,4), we used a batch of short-term tests to examine the effects of tea pigments on the initiation, promotion and progression stages during carcinogenesis. We also examined the inhibitory effect of tea pigments on liver and colorectal tumor formation in rats, and oral tumor formation in hamster. In addition, we investigated the effects of tea pigments on phase II detoxification enzymes to further elucidate their anti-tumor mechanism. Tea pigments samples were provided by the Institute of Tea Science and Research, Chinese Academy of Agricultural Sciences.

Methods
Short-term Screening Assays
Test on the induction of forward gene mutation by mitomycin C in V79 cells was based on the methods reported by Cajelli et al (5). Micronuclei formation in V79 cells induced by mitomycin C (MMC) was measured by the cytokinesis-block method as described previously (Krishna et al)(6). Metabolic cooperation in V79 cells induced by TPA was based on the method reported by Yitti et al(7). Test on TPA induced inflammatory epidermal edema in mouse ear was carried out according to the method described by Gschwendt(8). Tests on the viability of Hela cells and growth ability of cells in soft agar test were based on the methods reported by Wu (9). Test on the growth of S-180 solid tumor in Kunming mice were conducted following Yamaguchi et al (10).
Quinone Reductase Activity Assay

Hep G2 cells were grown for 24 hours in a humidified incubator in 5% CO₂ at 37°C. After 24 hours treatment, the cells were lysed by incubation at 37°C for 10 min with 50 ml in each well of solution containing 0.8% digitonin. The number of cells or the amount of protein in each microtiter was determined by the method of Delong (11).

Rat Model of Liver preneoplastic lesions (12)

Male Wistar rats, 80-100 g b.w., were divided into 3 groups randomly. The positive-control group was treated as follows: given nitroso-diethylamine (NDEA) (10 mg/kg b.w.) by i.p. once a day from day 7 to 13, 20% CCl₄ (0.5 ml/rat) by i.p. once a day at day 40 and 41, and then subjected to 2/3 hepatectomy under ether anesthesia at the end of sixth week. The rats were killed at day 56. Cytosolic GST activity was analyzed based on the rate of GS-CDNB conjugate formation using CDNB as substrate as described by Habig (13). GST Isoenzymes were analyzed by Western Blotting.

Rat colorectal cancer model

One hundred and twenty six weaning male Wistar rats were randomly divided into three groups, 42 rats in each group. Rats in group 1 were given s.c. injection of dimethylhydrazine (DMH-2HCl) once weekly for 10 weeks at a dose of 20mg/kg body weight. Animals in group 2, in addition to the same carcinogen treatment as in group 1, 0.1% tea pigments as the sole source of drinking fluid. Animals in group3 were injected s.c. with equal volumes of physiological saline. Animals were killed at weeks 16 and 32. The number of tumors were counted and the length (L), width (W) and height (H) of each tumor were measured to calculate the tumor volume (V= LxWxHxπ/6). ACF were quantified following the protocol established by McLellan and colleagues (15). Two deparaffinized sections were used for PCNA staining by using a Straptavidin/Peroxidase (SP) kit (ZYMED) (16). The expression of ras-p21 was estimated using the same method as the determination of GST isoenzymes. Bcl-2 and Bax were stained using the same method as PCNA staining.

Hamster oral cancer model

Seventy-four male golden Syrian hamsters were randomly divided into five groups. The animals in group 1 were treated three times per week with DMBA. The animals in groups 2, in addition to the DMBA application as in group 1, received 0.1% tea pigments as the sole source of drinking water for two weeks before starting the DMBA treatment and until the end of the experiment. In the negative control group, same amount of acetone was applied topically to the right pouch. All hamsters in group 1-3 were sacrificed after 15 weeks of DMBA treatment. The tumor volume was calculated by the formula: vol = 4/3πr³. Mean tumor burden was determined by multiplying the number of tumors in each group by the mean tumor volume in millimeters. Two deparaffinized sections were used for AgNORs analysis, using one-step silver colloid method for AgNORs staining (17). The method described by Wargovich et al (18) was used.

Immunohistochemical analysis of EGFR was used the same method as the analysis of Bcl-2.

Results

Effects of tea pigments in short-term tests

Tea pigments exhibited inhibitory effects on forward gene mutation at the concentration of 10-100 mg/ml and the inhibitory effect has significant dose-response relationship (p<0.01). The inhibition rates were 43.9% and 76.7%. The same pattern of inhibition was observed on the micronuclei formation in V79 cells induced by mitomycin C. Inhibition was significant at the concentration of 10-100 mg/ml of tea pigments with dose-response relationships. The results of tea pigments on the metabolic cooperation test showed that when V79 cells were mixed with M cells, the survival rate of M cells was significantly reduced, because of the occurrence of metabolic cooperation. However, when TPA was added to the system, the metabolic cooperation was inhibited and the relative survival rate of M cells was recovered. When tea pigments were added at concentrations ranging from 10 to 20 mg/ml with TPA, the survival rate of M cells was reduced. Dose-response relationship was observed as the concentration increased. In the test of inflammatory epidermal edema induced by TPA, tea pigments alleviated the degree of edema. The effect of tea pigments showed significant dose-response relationship. Tea pigments have significant inhibitory effects on the survival rate and grow ability of Hela cells in soft agar medium. At the concentration of 50 mg/ml, the inhibitory rates of Hela cells viability were 22.1% respectively. At the concentration of 100 mg/ml, the inhibitory rates of Hela cells growth were 34.1%.

Effects of tea pigments on detoxication enzymes.

Tea pigments increased QR activity in Hep G2 cells 60.4% by theaflavins and thearubigins. Oral
administration of 0.1% tea pigments as drinking water induced cytosolic GST activity by 18% in rat liver with precancerous lesions induced by NDEA as compared with the positive control group. At the same time, GST 1-1, 1-2, and 3-3 protein expression level in liver cytosol of these two groups were also increased.

**Rat colon cancer**

ACF were formed in DMH-treated rats but there was no ACF formation in the negative control group. Compared with the positive control group, the total number of ACF per colon in rats was significantly decreased ($P<0.01$). Small ACF, medium ACF and large ACF in group 2 were also significantly reduced.

At the end of the experiment, all rats in the positive control group developed colonic tumor while no tumors were developed in the negative control group. Animals that consumed 0.1% tea pigments had significantly fewer tumors than that of the positive control group, and the mean volume was also dramatically lower.

After 16 and 32 weeks, the PCNA-LI in DMH-treated groups were significantly increased over the negative control group. Oral administration of 0.1% tea pigments decreased the PCNA-LI ($P<0.01$). The expression of ras-p21 in colonic mucosa and tumors were significantly decreased as compared with the positive control group ($P<0.01$). Bcl-2 expression was significantly suppressed in tea pigments-treated group as compared with the positive control group, while Bax expression was significantly induced in tea pigments-treated group ($P<0.05$ or $P<0.01$).

**Hamster Oral Tumor**

At the end of 15 weeks, all the hamsters in the positive control group developed oral tumor and carcinoma, while the animals in the negative control group did not develop any oral tumor. Animals that consumed 0.1% tea pigments had significantly fewer tumors and carcinoma than that of the positive control group ($p<0.01$), and the mean volume and the mean tumor burden was also dramatically decreased ($p<0.01$). When compared with the positive control group, the mean tumor burden was reduced by 89% and the incidence rate of carcinoma was reduced by 38.5% in the tea pigments group ($p<0.01$). At the same time, the incidence of micronucleated cells, the number of AgNOR dots per nucleus TVNOR, the labeling index of PCNA and EGFR expression in the buccal mucosa in tea pigments-treated were noticeably increased over the negative control group ($p<0.01$). Oral administration of 0.1% tea pigments significantly decreased the micronuclei formation, the number of AgNOR dots per nucleus, TVNOR, the labeling index of PCNA and EGFR expression ($p<0.01$).

**Discussion**

In short-term tests, tea pigments significantly inhibited the forward gene mutation and micronuclei formation in V79 cell induced by mitomycin C, V79 cells metabolic cooperation and survival and growth ability of Hela cells in soft agar media. Tea pigments showed a strong inhibitory effect on S180 solid tumor formation in mice. These results are consistent with the results of black tea's effect on carcinogenesis reported previously by our group (19).

The results on Hep G2 cell assay showed that tea pigments could induce the activity of antioxidant enzymes and phase II enzymes, i.e. QR and GST, thus enhance detoxification of carcinogen in vivo. In the QR activity assay, we found that the effects of theaflavins or thearubigins are weaker than the effect of tea pigments. It is concluded that the effect of individual components in tea pigments is not as strong as the mixed form tea pigments.

Tea pigments was effective in the prevention of carcinogenesis in rat liver and colorectal cancer model as well as hamster oral cancer model. The results suggested that tea pigments is protective in a wide spectrum of animal carcinogenesis models.

The following mechanisms are suggested based on the above results in terms of the protective effects of tea pigments on cancer formation. (1) to protect DNA damage, which is shown by the inhibition of micronuclei formation in hamster oral cancer model. (2) to inhibit the proliferation of cancer cells. (3) to induce apoptosis, which is shown by the inhibition of Bcl-2 and the induction of Bax protein. (4) to inhibit the expression of related oncogenes, such as ras oncogene.

Although the results reported in the paper provide strong evidence on the protective effects of tea pigments on cancer formation in various animal models, human evidence from intervention trials is needed.

**Reference**


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